PREPARATION, CHARACTERIZATION AND SOME BIOLOGICAL ACTIVITIES OF WATER SOLUBLE CHITOSAN DERIVATIVES

By

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DEDICATION

TO the memories of my beloved mother (SAYDAH)

TO my dear father

TO my husband and my children

TO my sisters and brothers

ACKNOWLEDGMENTS

Firstly, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

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LIST OF SYMBOLS

ppm	Parts-per-million
λ	Wavelength
cm	Centimetre
T	Temperature
µg	Microgram
˚C	Degree Celsius (centigrade)
mg	Milligram
kDa	Kilodalton
W	Mass
H	Hour
V	Volume
β	Beta
a.u	Arbitrary unit for intensity
nm	Nanometre
mL	Millilitre

LIST OF MAJOR ABBREVIATIONS

APS	Ammonium persulphate
C-1-6	Carbon number 2, 3, 4, 5 and 6
CAN	Ammonium cerium (IV) nitrate
CCD-18	Normal Fibroblast
CLSI	Clinical and Laboratories Standards Institute
COSY	Correlation spectroscopy
CS-2AP	2-Aminopyridine chitosan
CS-AM	Polycrylamide chitosan
CS-APAM	2-Aminopyridine polyacrylamide chitosan
CS-APDCDA	2-Aminopyridine dicyandiamide chitosan
CS-BCOE	Bis (2-chloroethyl) ether chitosan
CS-DCDA	Dicyandiamide chitosan
CS-HQ	8-Hydroxyquinoline chitosan
CS-MEOXANAM	4-Methoxy aniline polyacrylamide chitosan
CS-mPhenol	4-Methyl phenol chitosan
CS-OXAM	4, 7, 10-Trioxa-1, 13-tridecanediamine chitosan
CS-PhAL	L- phenylalanine chitosan
CS-PhALAM	L-Phenylalanine polyacrylamide chitosan
CS-RES	Resorcinol chitosan
CS-RESAM	Resorcinol polyacrylamide chitosan
CS-VPDMAEM	Poly (1-vinyl pyrrolidone-co-2-dimethyl amino ethyl methacrylate) chitosan
DA	Degree of acetylation
DD	Degree of degradation
DMSO	Dimethyl sulfoxide
DOX-GC	Doxorubicin conjugated glycol chitosan
DPPC	Dipalmitoyl phosphatidylglycerol
DSC	Differential scanning calorimetry
E. coli	*Escherichia coli*
EAT	Ehrlich ascites tumor
EPR	Enhanced permeability and retention

FT-IR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
HCT-116	Colon cancer cell line
HepG2	Human liver hepatic cellular carcinoma cell line
HIFBS	Heat-ina ctivated fetal bovine serum
HMQC	Heteronuclear multiple-quantum correlation
IC50	Median inhibitory concentration
KPS	Potassium persulphate
L1210	Murine leukemia
M5076	Murine liver metastatic tumor
MAS	maleic acid sodium
MCF-7	Breast cancer cell line
Md	Molecular weight of organic compounds before reacted with chitosan
MH134	Murine hepatic cell carcinoma
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MMC	Mitomycin C
Mn	Number-average molecular weight
MTT	(3-(4, 5-dimethylthiazol-I2-yl) - 2,5 diphenyl tetrazolium bromide)
Mw	Weight-average molecular weight
Mz	Size-average molecular weight
Mz/Mw	Polydispersity index
NMR	Nuclear magnetic resonance
OM	Outer membrane
PC3	prostate cancer line
PEG	Polyethylene glycol
PL	Photoluminescence
RAW 264.7	Mouse monocyte macrophage
S. aureus	*Staphylococcus aureus*
SEM	Scanning Electron Microscopy
SMMC-7721	Human hepatocellular carcinoma cells

T_g	Glass transition temperature
TGA	Thermogravimetric analysis
XRD	X-Ray diffraction

PENYEDIAAN DAN PENCIRIAN TERBITAN KITOSAN TERLARUT AIR DENGAN BEBERAPA AKTIVITI BIOLOGI

ABSTRAK

Tujuan penyelidikan ini adalah untuk mensintesis terbitan kitosan larut air yang baru, serta mengkaji sifat fizik dan sifat kimia, mereka disamping sifat antibakteria dan antikanser. Justeru, 14 terbitan kitosan disintesis dengan julat berat molekul yang luas (daripada 0.56 hingga 220 kDa). Pelbagai kumpulan berfungsi, iaitu fenilanilina, benzena-1,3-diol, metilfenol, 8-hidroksikuinolina, 2-aminopiridina, (bis(2-kloroetil) eter, 4,7,10-trioksa-1,13-tridekanadiamina, disiandiamida, akrilamida dan poli(1-vinilpirolidon-ko-2-dimetilaminoetilmetakrilat telah digunakan untuk menghasilkan kitosan yang terubahsuai. Terbitan ini dicirikan dengan menggunakan pelbagai teknik alatan seperti spektroskopi inframerah transformasi Fourier (FT-IR), spektroskopi resonan magnetik nuklear (NMR) (proton dan karbon-13), analisis termogravimetrik (TGA) dan kalorimetri pengimbasan pembezaan (DSC). Kebanyakan terbitan ini menunjukkan keterlarutan dalam keadaan neutral dan berasid. Terbitan kitosan menunjukkan darjah pendeasetilan yang lebih rendah daripada kitosan, dan keadaan hablur kitosan bertukar kepada amorfus selepas kumpulan berfungsi diperkenalkan. Kebanyakan terbitan juga menunjukkan sifat pendarcahaya yang menarik. Semua terbitan mempunyai kesan antiproliferatif yang berbeza terhadap tiga jalur kanser apabila ujian *in vitro* dijalankan, iaitu kanser prostat, kolon dan kanser payudara pada pH neutral. Terbitan 4,7,10-trioksa-1,13-tridekanadiamina kitosan menunjukkan ketoksikan yang rendah terhadap sel kanser payudara dengan IC_{50} = 109.8 µg mL^{-1}. Bis(2-kloroetil) eter kitosan menunjukkan aktiviti yang signifikan terhadap kanser payudara MCF-7 dengan IC_{50} = 57.25 µg

mL^{-1}. 8-Hidroksikuinolina kitosan menunjukkan kesan antiproliferatif yang amat tinggi terhadap jalur sel kanser yang diuji, iaitu jalur sel kanser prostat PC3 dengan IC_{50} = 12.11 µg mL^{-1}, jalur sel kanser payudara MCF-7 dengan IC_{50} = 14.18 µg mL^{-1} dan jalur sel kanser kolon HCT-116 dengan IC_{50} = 79.21 µg mL^{-1}. Sifat antibakteria terhadap bakteria gram-negatif, iaitu *E. coli* dan bakteria gram-positif, iaitu *S. aureus* menunjukkan. Kepekatan Perencatan Minimum (MIC) pada 1000 µg mL^{-1}. Namun demikian, kebanyakan terbitan kitosan menunjukkan nilai MIC yang lebih rendah, iaitu dari 500 hingga 62.5 µg mL^{-1}.

PREPARATION, CHARACTERIZATION AND SOME BIOLOGICAL ACTIVITIES OF WATER SOLUBLE CHITOSAN DERIVATIVES

ABSTRACT

The aim of this research is to synthesise novel water-soluble chitosan derivatives and to investigate their physical, chemical as well as antibacterial and anticancer properties. Thus, fourteen chitosan derivatives were synthesised with a wide range of molecular weights (from 0.56 to 286 kDa). Various functional groups, i.e. phenylaniline, benzene-1,3-diol, methylphenol, 8-hydroxyquinoline, 2-aminopyridine, (bis(2-chloroethyl)ether, 4,7,10-trioxa-1,13-tridecanediamine, dicyandiamide, acrylamide, poly(1-vinylpyrrolidone-co-2-dimethylaminoethyl methacrylate) were used to produce the modified chitosans. These derivatives were characterized using various techniques such as Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) (proton and carbon-13) elemental analysis, thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). Most of these derivatives showed remarkable solubility in neutral and acidic conditions. The chitosan derivatives showed lower degree of deacetylation than chitosan, and the crystalline state for chitosan was changed to amorphous after the introduction of the functional groups. Most of the derivatives also showed interesting luminescent properties. All derivatives have different antiproliferative effect against three cancer lines when tested *in vitro* namely prostate, colon and breast cancer at neutral pH. The 4,7,10-trioxa-1,13-tridecanediamine chitosan derivative exhibited mild cytotoxicity against breast cancer cell with IC_{50} = 109.8 µg mL^{-1}. Bis(2-chloroethyl) ether chitosan showed significant activity against breast cancer MCF-7 with IC_{50} = 57.25 µg mL^{-1}. 8-

Hydroxyquinoline chitosan exhibits the highest antiproliferative effect against the tested cancer cell lines i.e., prostate cancer cell line PC3 with IC_{50} = 12.11 µg mL^{-1}, breast cancer cell line MCF-7 with IC_{50} = 14.18 µg mL^{-1} and colon cancer cell line HCT-116 with IC_{50} = 79.21 µg mL^{-1}. Antibacterial properties against gram-negative bacteria namely *E. coli* and gram-positive bacteria namely *S. aureus* showed Minimum Inhibitory Concentration (MIC) at 1000 µg mL^{-1} for chitosan. However, most of the chitosan derivatives showed lower MIC values namely from 500 to 62.5 µg mL^{-1}.

CHAPTER 1

INTRODUCTION

1.1 Chitosan

Chitosan is a natural polysaccharide polymer that is obtained from the partial deacetylation of chitin (Figure 1.1.a) [1]. Chitosan are high-molecular-weight and linear heteropolysaccharide compound that composed of a β (1→4) linked to two monosaccharides, N-acetyl-D-glucosamine and D-glucosamine. The structure of chitosan can be observed in Figure 1.1.b. Chitosan consists of three types of reactive functional groups, which are an amino group at C-2, primary hydroxyl group at C-3 and secondary hydroxyl at C-6 positions, respectively [2, 3].

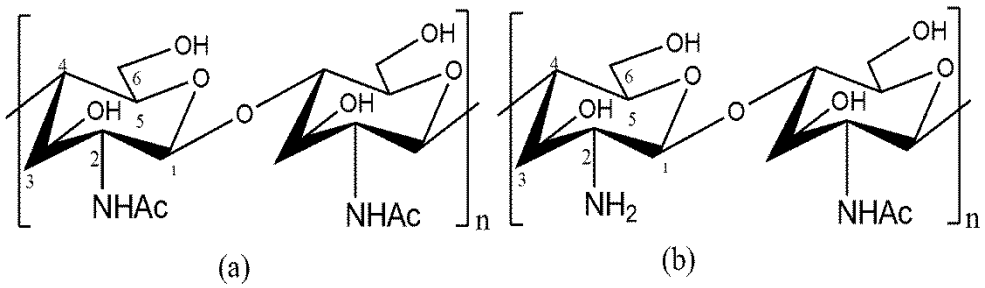


Figure 1.1 Structures of (a) chitin and (b) chitosan

In 1859, Rouge discussed the deacetylated form of chitosan by subjecting chitin to alkali treatment [4-6]. The interest on the use of chitosan has continued between 1930s to early 1940s [7]. In 1960s, a number of Asian nations such as Japan, Korea, Singapore, Taiwan, and Thailand were able to achieve ample amount of research that focused on better understanding of chitin and chitosan materials, and covering the whole spectrum of research from better production to the purification methods [5]. In 1970s, there was a resurgence of interest in chitosan [5, 6]. China has

joined these researchers and become an important producer of chitin and chitosan in Asia [5]. In the last few decades, chitosan has shown as a potential source of bioactive material and abundant of applications [8].

1.1.1 Production of Chitosan

The production of chitosan starts from the isolation of chitin from shells of crustaceans wastes of the seafood industries such as (shrimp, crab, and crawfish) (Table 1.1) [9,10]. These natural sources produced chitin amounting to a hundred billion tons each year [11].

Table 1.1: Contents of chitin from natural resources as reported by Kurita [10]

Source	Chitin (%)
Crab cuticle	15–30
Shrimp cuticle	30-40
Krill cuticle	20–30
Squid pen	20–40
Clam/oyster shell	3-6
Insect cuticle	5–25
Fungi cell wall	10-25

These wastes from the seafood industries composed of lipids as primary structural components, protein, inorganic salts, and chitin. They are initially crushed and filled as powder to initiate further heterogeneous processes (Figure 1.2) [9].

In the first approach, 5 % sodium hydroxide was added to the shells to dissolve the various proteins, leaving behind chitin, lipids and calcium salts (mainly as $CaCO_3$). Then the mixture was treated with 30 % hydrochloric acid to dissolve the calcium salts (demineralization). This step also hydrolyzes the lipids, dissolves other minor inorganic constituents and produces chitin. The obtained chitin is hydrolyzed at 100 °C in the presence of 50 % sodium hydroxide solution to produce chitosan [9].

In the second approach, 5 % hydrochloric acid was added to the shells powder to remove calcium salts. This step followed high-temperature (110 °C) treatment with 40 % sodium hydroxide to remove protein and lipids. During the treatment, a concomitant hydrolysis of acetamide groups in chitin has taken place, resulting in the formation of chitosan [1, 9].

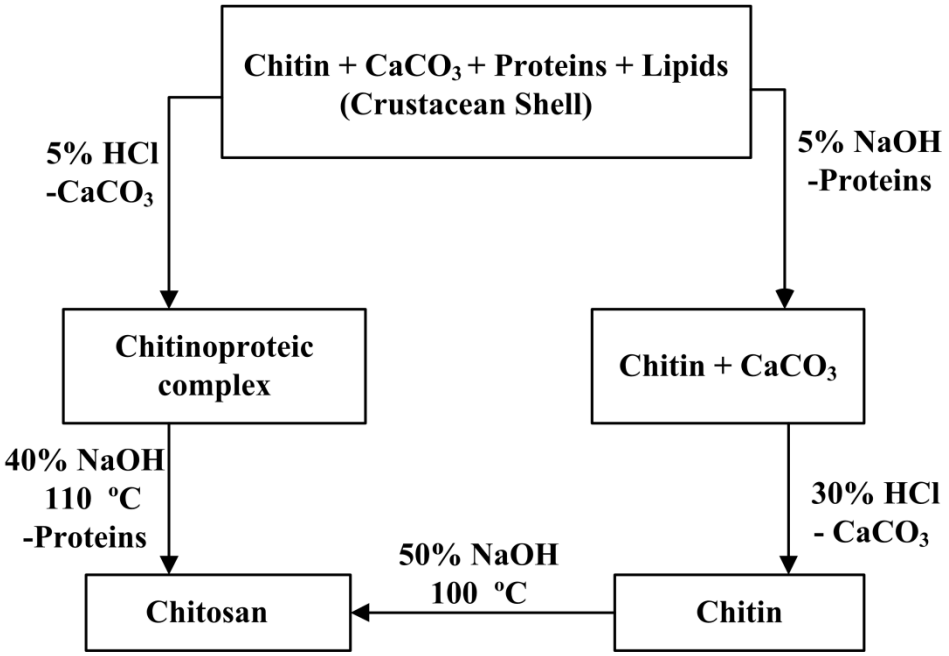


Figure 1.2 Isolation of chitin and synthesis of chitosan as reported by Olteanu [9]

1.1.2 Properties of Chitosan

The characteristics of chitosan and the final product performance are affected by many conditions such as the type and concentration of reagents, the time and the reaction temperature throughout the processing. The relevant parameters of the product are degree of deacetylation (DD) and molecular weight that influences the solubility, rheological and physical properties [8]. The variation in commercial chitosan grades is due to the difference in the DD and molecular weight. However,

the improvements in chitosan characteristics can be achieved through modifications of the DD and the molecular weight. For example, the value of DD can be decreased by deacetylation [12, 13] while the molecular weight can be reduced by acidic or enzymatic depolymerization [14, 15].

1.1.2.1 Molecular Weight

Molecular weight directly influences the chemical, physical and biological properties of chitosan, such as viscosity, solubility, adsorption on solids, elasticity and tear strength [8]. The effect of chitosan for biomedical applications comes not only from their chemical structure but also comes from the molecular weight [15]. It was observed that the crystallinity of chitosan increased with decrease in molecular weight [8]. The average molecular weight can range from 50 to 2000 kDa, depending on the preparation processes and the source [16, 17].

1.1.2.2 Degree of Deacetylation

The degree of deacetylation (DD) can be used to specify the variation of acetyl content for this family of polymer. The names chitin and chitosan correspond to acetyl content that is measured by the DD [8]. Deacetylation is the removal of acetyl groups from the chitin molecular chain, leaving behind amino groups (NH_2). Increasing the number of this amine group in chitosan requires repeated alkaline treatment to achieve high deacetylation (can be up to >90% [13]). Increasing the number of amine groups can also be done by increasing the temperature or the strength of the alkaline solution during the deacetylation reaction [8]. Its property is dependent on the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose in the structural units and the content of free amino groups

and can be used to differentiate between chitin and chitosan [1]. If the percentage of 2-amino-2-deoxy-D-glucopyranose units in the component is more than 50%, it is termed chitosan. Conversely, when the percentage of 2-acetamido-2-deoxy-D-glucopyranose units is higher, the term chitin is used [7, 18]. Nevertheless, in 2002 and Tommeraas et al. [19] suggested that chitin with DD of 60% and above to be known as chitosan.

The degree of crystallinity in chitin and chitosan is a function of the degree of deacetylation. High DD will result in chitosan with high crystallinity [16]. Solubility in acidic solution and the crystallinity of chitosan shows a strong association with the value of DD [20]. Chitosan with different ranges of DD (40 to 98%) has been commercially produced [16, 17].

1.1.2.3 Solubility of Chitosan

Chitosan is a weak base, which is insoluble in water, alkali or aqueous solutions at pH value higher than 7 and also in most organic solvents. Chitosan has the ability to dissolve in certain inorganic and organic acids such as hydrochloric, nitric, acetic , phosphoric, propionic, succinic, tartaric, lactic, citric and formic acids at certain pH values after prolonged stirring [8, 16, 21-23]. Chitosan is insoluble in sulphuric acid because they can react to form a white crystalline solid of chitosan sulphate [23]. The variation of chitosan solubility in acids depends on the pK_a of these acids and their concentrations [8, 20].

The solution viscosity in all solvent system is affected by polymer concentration, temperature, counter-ion concentration and pH [8]. For example, solution at pH below 4 postive charge of protonated amino group in chitosan causing to enhance the electrostatic repealing between charged groups of similar charge,

leading to promotion of swelling of the chitosan network [20]. While solvent with pH of 5.2 generates chitosan with the unstable structure because the free amino groups compose intermolecular hydrogen bonds with the oxygen in an excess of NaOH [24]. For solution at pH value higher than 6.5, which is roughly equal to the pK_a of chitosan amino group, the phase separation happens with the increase in aggregate size and the chitosan obtained coagulates and is recovered as an amorphous solid [20, 25]. The free amino (NH_2) groups in chitosan chain forms conformational features through intramolecular and/or intermolecular hydrogen bonding and make it a cationic polyelectrolyte ($pK_a \approx 6.5$), making it soluble in acidic solutions below pH ~ 6.5 [20].

The major limitation of chitosan comes from the poor solubility at physiological pH (7.4), especially for biomedical applications where it is insoluble and ineffective as an absorption enhancer [26]. There are many improvements of chitosan solubility such as by reducing the molecular weight [14, 15, 23]. Different chemical modification techniques that involve introducing a hydrophilic functional group such as polyethylene glycol (PEG) have been employed in previous studies to produce water-soluble derivatives of chitosan [16, 19, 20, 23, 26, 27]. The recently good water solubility of modified chitosan has attracted the attention of many researchers such as O-quaternary ammonium N-acyl thiourea chitosan [7].

1.1.2.4 Viscosity

The viscosity of chitosan is an important factor in determining its commercial and biological applications. The viscosity of chitosan is proportional to the molecular weight and conversely with the demineralization time [28]. Moreover, it is affected by other factors during production processes such as the degree of deacetylation,

concentration of the solution, ionic strength, pH, and temperature. The intrinsic viscosity of chitosan depends on the degree of ionization more than the ionic strength [29]. This is confirmed by the variation of chitosan viscosity in acids such as in acetic acid. The viscosities increases with decreasing pH, In case of decrease pH by using HCl causes to lower the viscosity. It means depend of kind of acid used to decrease the pH. Bough et al. [29] and Moorjani et al. [28] have reported that the production of chitosan with 3% NaOH or acetone considerable decreases the viscosity of the final chitosan products. They concluded that chitosan viscosity is strongly affected by physical treatments such as grinding, heating, autoclaving and ultrasonication and showed that the decrease in viscosity with an increase in treatment time and temperature [30].

Bough et al. [29] investigated the effect of particle size on the viscosity of chitosan. They found that smaller particle size (1 mm) resulted in chitosan products of both higher viscosity and molecular weight than those of either 2 or 6.4 mm particle size, because the larger particles need more swelling time.

1.1.2.5 Biological Properties of Chitosan

Chitosan and its derivatives have appealing biological properties such as low toxicity, biodegradability, and biocompatibility. These properties lead to commercial production of chitosan and its derivatives for medical, pharmaceutical and industrial applications [31, 32, 33].

1.1.2.5.1 Biodegradability

Biodegradability is one of the most essential and valuable properties of chitosan and its derivatives. Usually, non-toxic oligosaccharides lengths can be

obtained from the biodegradation of chitosan that can be generated by using several techniques such as physical, enzymatic and chemical methods. Physical techniques such as ozone treatment and ultraviolet radiation accelerate the degradation of chitosan [34]. Enzymatically, chitosan can be degraded in vitro, by several non-specific enzymes such as lysozymes, pepsin, papain, cellulase, pectinase, proteases and lipases that are found in mammals [35, 36]. The oligosaccharides can be subsequently incorporated into glycosaminoglycans and glycoproteins metabolic pathways [16]. It believed that chitosan degrades invertebrates mostly by lysozyme and bacterial enzymes inside the colon [37].

Chitosan degradation is affected by the DD value and the distribution of their acetyl groups. The DD value that controls the degree of chitosan crystallinity is inversely proportional to degradation kinetics while reducing of acetyl groups or their homogeneous distribution caused very low rates of enzymatic degradation [38, 39]. The association of chitosan degradation and the DD value has been investigated in several studies, and all of them obtained the same inversely proportional relationship [12, 40, 42]. In 2005, Kofuji et al. [41] studied the effect of enzymes on chitosan degradation. They considered various chitosans viscosity in the presence of lysozyme solutions; finally they reported that chitosan with low DD degraded more quickly. Other studies mentioned that the variation in chitosan degradation can be attributed to the differences in the distribution of acetamide groups in the chitosan molecule [38, 41]. Differences of this distribution changes inter or intramolecular repulsion forces, which influences the viscosity of the chitosan solution [12].

1.1.2.5.2 Biocompatibility

Biocompatibility is one of the most attractive characteristics of chitosan. For example, the role of chitosan is not changed by host medium conditions and there are no any undesirable local or systemic effects have been recorded or observed due to changes in function of chitosan. In spite of the living tissues, including the skin, ocular membranes and the nasal epithelium showed real acceptance or well tolerated to chitosan. Biocompatibility characteristics also depend on the property of the origin of chitosan such as natural source, method of preparation, molecular weight (Mw), and DD [12].

1.1.2.5.3 Toxicity

Chitosan has very low toxicity when compared with other natural polysaccharides. Chitosan showed lethal dose LD50 around 16 g kg^{-1}, very close to the value obtained for salt and glucose carried out in the laboratory by *in vivo* toxicity assays [42]. In an earlier study, it was found that the toxicity of chitosan is strongly correlated with the DD value. It is reported that chitosan with DD values higher than 35% exhibited low toxicity while chitosan with DD value under 35% showed dose-dependent toxicity [34].

1.2 Previous Studies on Modification and Applications of Chitosan

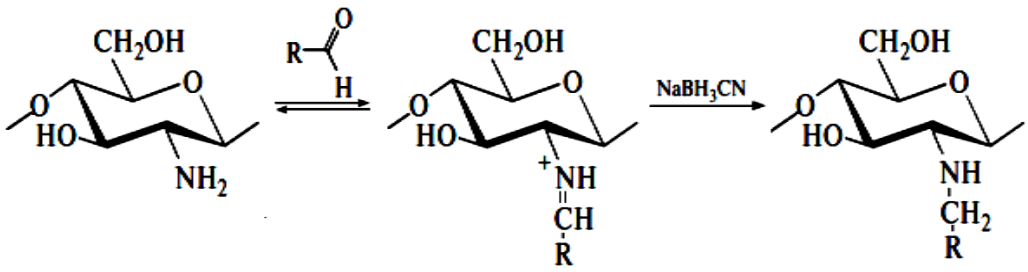
This section presents the previous studies and survey of the relevant literature on the chemically modified chitosan and its applications.

1.2.1 Chemically Modified Chitosan

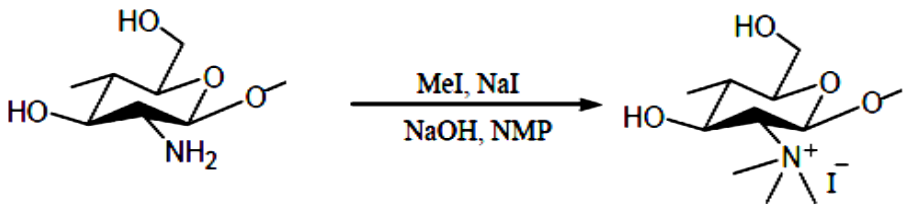
Chemically modified chitosan has been extensively explored and can be considered as the most important method to improve the water solubility of chitosan by adding some hydrophilic functional group [43, 44]. The synthetic methods of chitosan derivatives reported in the literature falls under one of the three categories; N-alkylation, N and/or O acylation and grafting of chitosan.

1.2.1.1 Alkylated Derivatives of Chitosan

N-alkylated chitosan has been prepared by reacting aldehydes or ketones (the Schiff reaction) with the amino groups of chitosan. Then addition of borohydride $NaBH_4$ or sodium cyanoborohydride $NaBH_3CN$ to reduce imines formed to amines as shown in Scheme 1.1 [44 - 46]. It has also been reported that N-alkylated chitosan could be obtained by the reaction of alkyl bromide [47] or alkyl iodide [48] with amino groups of chitosan, leading to quaternary ammonium formation as shown in Scheme 1.2. Domard et al. [49] used this approach to generate a wide pH range of water soluble cationic derivatives.



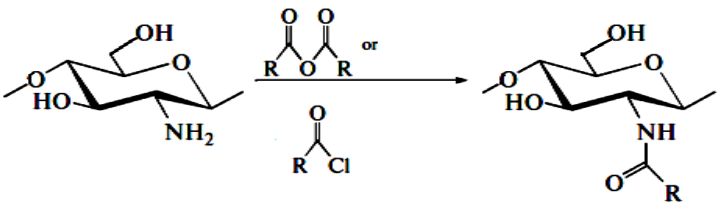
Scheme 1.1: N-alkylation of chitosan using reductive amination approach



Scheme 1.2 Quaternization of chitosan with methyl iodide

1.2.1.2 Acylation Derivatives of Chitosan

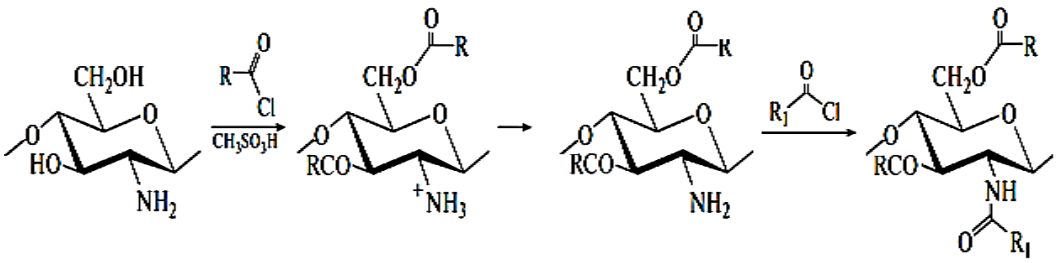
Derivatization by acylation has more synthetic pathways compared to N-alkylation because hydrophobic groups can react with N or O atoms or to both atoms at the same time. Aranaz et al. [45] reported that the derivatization of chitosan by a hydrophobic group is formed via an ester bond that can be considered as the enzymatic actions. They noted that the chitosan derivatives were randomly distributed along the chain, through alkylation or acylation under homogeneous conditions. Various works were successful in the preparation of N-acylated derivatives of chitosan through the anhydrides, acid chlorides interaction with the amino groups of chitosan as shown in Scheme 1.3.



Scheme 1.3: N-acylated derivatives of the chitosan formed through the anhydrides, acid chloride interaction

In the preparation of O substituted derivatives, it is necessary to keep the amino group in positive charge thus it works well in an acidic solution. The activity of amino groups is higher than the hydroxyl groups, consequently amino groups are blocked prior to O acylation, such as in the reactions with acid chlorides in methane sulfonic acid as shown in Scheme 1.4 [50]. N, O acylated chitosan reported in prior works followed by steps procedure, the first step chitosan O acylation by blocking amino groups. The second step involves protection of amino groups removed and continued with N-acylation synthesis [45, 51, 52]. Riva et al. [53] and Aranaz et al. [45] described the synthesis of N; O acylated chitosan compounds in their reviews.

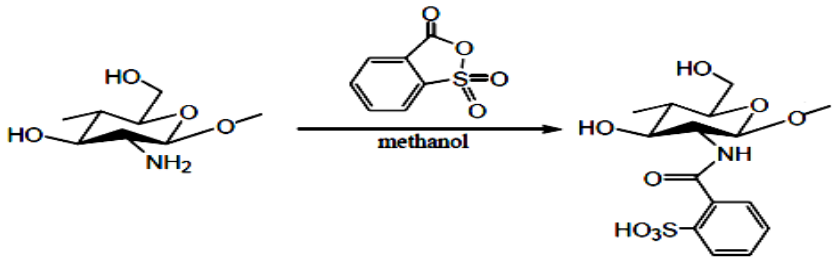
They refer to various methods that have been used to prepare hydrophobic derivatives of chitosan by the combination of hydrophobic groups into chitosan chains.



Scheme 1.4: N, O acylated chitosan by the combination of hydrophobic groups

1.2.1.3 Thiolation of Chitosan

Anionic charge can be introduced to the basic structure of the chitosan polymer, by the reaction of chitosan with 2-sulfobenzoic acid anhydride as shown in Scheme 1.5 [54]. Jayakumar [55] reported the chemical modification of chitosan with sulfate produced novel bifunctional materials that are promising for biomedical applications.

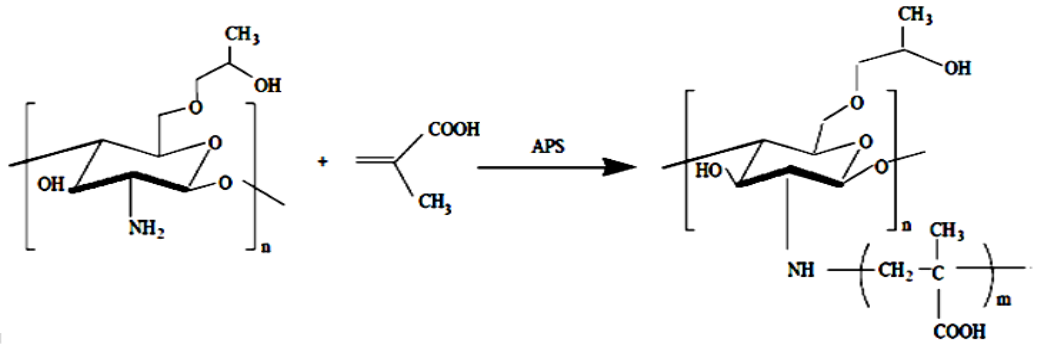


Scheme 1.5: N sulfonation of chitosan with 2-sulfobenzoic acid anhydride

1.2.1.4 Grafting of Chitosan

Many studies have been carried out on the grafting of polysaccharide based materials to improve properties for the biomedicine and environmental field of applications [56, 57]. Several investigations have been reported on the study of

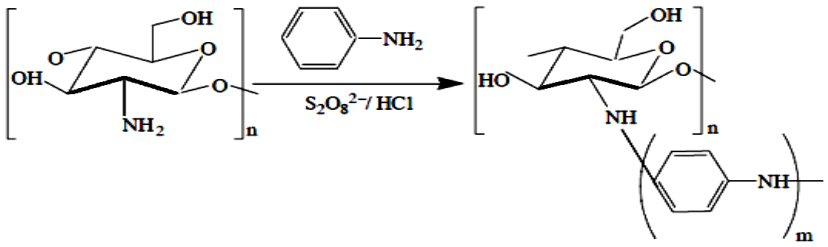
grafting natural polymers by using radiation for example as reported by Jaafar et al. [58] and Pengfei et al. [59]. Enzymatic grafting in several previous works described some advantages of synthesis and modification of polymers by using enzymes [60]. Ilyina et al. [61] reported that enzymatically modified polymers such as partially hydrolyzed chitosan can be obtained with similar biochemical properties compared to chitosan. Chitosan has also been grafted through the addition of cationic polymerization as reported by Yoshikawa et al. [62] They have performed grafting reaction by introducing poly (isobutyl vinyl ether) and poly(2-methyl-2-oxazoline) cation onto the amino groups in the chitosan. Recently, grafting of vinyl monomers through free radical initiation has attracted the interest of several researchers. This approach offered wider options with new structures and preferred properties. Sun et al. [63] used ammonium persulphate (APS) as an initiator in an aqueous solution to prepare carboxymethyl chitosan grafted methacrylic acid. Similarly, Xie et al. [64] succeeded in producing hydroxypropyl chitosan grafted methacrylic acid as shown in Scheme 1.6, which exhibited more improvement in water solubility.



Scheme 1.6: Graft copolymerization of methacrylic acid on hydroxypropyl chitosan.

Sun et al. [65] used APS initiator to graft copolymerization of maleic acid sodium (MAS) onto carboxymethyl chitosan and hydroxypropyl chitosan and reported that the reaction conditions played a critical role in the grafting

copolymerization. The polymerization of aniline was obtained by the reaction of aniline in the presence of APS with chitosan. In this reaction (Scheme 1.7) the polyaniline side chains are bonded with the chitosan amino groups [66].



Scheme 1.7 Reaction of chitosan in aqueous acidic medium with using APS as initiator [66]

Graft copolymerization of vinyl monomers onto chitosan is also carried out using redox initiator systems, such as ceric ammonium nitrate (CAN) and potassium persulphate (KPS). This approach was reported for the first time in 1958 by Mino and Kaizerman. [67] They employed a ceric ion redox initiating system to achieve copolymer grafting. After that, this approach witnessed magnificent development [68] especially in the case of cellulose [69] and starch [70]. Usually, this method produced grafted copolymers with medium to the high molecular weight that are spaced along the backbone of the polysaccharide [69].

In previous studies, some organic compounds were reacted with chitosan, such as amino acid and amino acid derivatives, aniline, phenol, ether and heterocyclic to modify chitosan. Layek and Singh [71] synthesized n-acetylamino and amino acids through coupling reaction of mediated carbodiimide which can be used in gene treatment. Zhang et al [72] introduced an oral delivery method of insulin by using N-octyl-N-arginine chitosan micelles. Yamada [73] has grafted hexyloxyphenol onto chitosan by using an enzymatic method. Yang and Yuan [74]

synthesized chitosan hydroxyl azacrown ether from the reaction of hydroxyl azacrown ether with epoxy-activated chitosan. Many heterocyclic compounds have been reacted with chitosan as reported by Badawy [75], such as heterocyclic aldehydes containing 3-pyridine carboxaldehyde, 5-methylfuran-2-carbaldehyde, furan-2-carbaldehyde and benzo[d] (1,3). The various techniques to prepare chitosan derivatives are shown in Figure 1.3 [76].

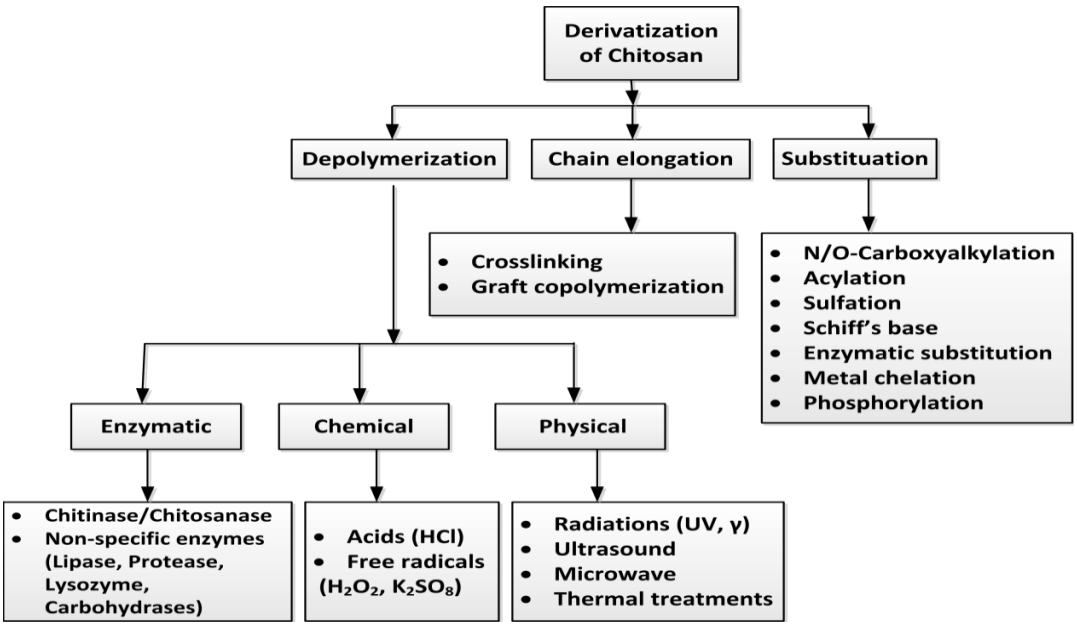


Figure 1.3: Techniques for derivatization of chitosan [76]

1.3 Applications of Chitosan

Chitosan and its derivatives have different practical properties, which make them appropriate for many applications such as in agriculture, food, wastewater management, cosmetics, fiber and biomedicine [7, 9]. The wide variation of chitosan molecular weights and degree of deacetylation, give opportunities to use it in different cosmetic fields such as skin care, water-resistance, sun protection, deodorants and hair care [5, 25]. Chitosan is also used for biomedicine applications; clinical studies have found 3-6 grams per day of chitosan can be used with no

adverse effects [9, 64]. Therefore, chitosan attracts lots of attention for the pharmaceutical formulation and different medical applications in topical and ocular applications, implantation or injection focusing on its biocompatible, absorption-enhancing, controlled release and bioadhesive properties [5]. Furthermore, chitosan can be considered as biodegradable, metabolized by some human enzymes, such as lysozyme and bioadhesive [77]. Carreno-Gomez [78] reported that chitosan extensively used for oral and intranasal delivery parenteral drug delivery. Chitosan has been used as starting material for man-made fibers, filament, powder, granule, sponge, absorbable sutures in medical products, composite with cotton or polyester and wound dressing materials. It could be considered for skin replacement [79] and antitumor effect through the proliferation of cytolytic T-lymphocytes [1, 32].

Chitosan can attach itself to fat in the stomach before it is digested, and it has no calorie value, making it good fat trapper for a weight loss products [1, 7]. Additionally chitosan and its derivatives have been used in the large area of biological fields such as an antibacterial, antifungal agent, to suppress viral infections in various biological systems, plants, animals and humans. Chitosan is also used for wound dressing materials [1, 7, 22, 23].

1.3.1 Anticancer Properties

Chitosan has direct effects on cancer cells by interfering with cell metabolism, stop cell generation, or stimulate cell apoptosis. It plays antitumor role by developing the resistant and protections of body functions. Many previous works *in vitro* and *in vivo* suggested that chitosan is very appropriate for complementary antitumor for drug and drug delivery applications [80, 81]. These studies discovered the significant changes in the antitumor activity of chitosan nanoparticles from

variable manufacturers and the quality of chitosan nanoparticles for particular cancer cell [82]. Maeda and Kimura [80] reported that chitosan with low molecular weight and chitosan oligosaccharide successfully stopped growing cancer cells in Sarcoma 180 - bearing mice. Torzsas et al. [81] have established that a diet including chitosan can decrease the growth of the cells containing precancerous lesions especially in colon cancer caused by azomethane compounds. In an other study, it was found that inhibition rates for different types of cancer cell lines (23 % of liver SMMC-7721 cells, 27 % of Hela cells of cervical cancer, 7721 cells, 29 % of gastric cancer BGC-823 cells 23 % on liver SMMC), and as high as 55 % of breast cancer MCF-7 cells, when treated with 500 mg L^{-1} chitosan nanoparticles [83].

Nanoparticles of carboxymethyl chitosan with high toxicity can be grafted from doxorubicin/methoxy polyethylene glycol (PEG). These nanoparticles have been found to stop the distribution of cancer cells effectively [84]. In addition, nanoparticles of paclitaxel chitosan have also exhibited sustained release influence with good encapsulation percentage of 94.0% ± 16.73% [85]. Qi et al. [86] have applied high surface charged nanoparticles of chitosan to human hepatoma BEL7402 cells *in vitro* and found that dose and time correlation with anticancer activity. Hosseinzadeh et al, [87] prepared nanoparticles of gemcitabine-loaded via an ionic gelation technique, by using Pluronic RF-127 with chitosan nanoparticles as a carrier of cytotoxicity on HT-29 colon cancer cell line and found the inhibition of cancer cell on HT-29 colon carcinoma cell. Similarly, chitosan–silica hollow nanospheres were studied (CS–SiO_2 HNPs) for breast tumor cells MCF-7 treatment *in vitro* and *in vivo* environments with high therapeutic effectiveness [88]. Ignacak et al. [89] reported the microcrystalline structure inhibition of Ehrlich ascites tumor (EAT) cells. Huang et al. [90] reported that cytotoxicity on human lung carcinoma cell line

A549 (*in vitro*) showed insignificant effect when molecular weight of chitosan is decreased. Jiang et al. [91] confirmed that tailored chitosan can stop cell production and make apoptosis in MCF-7 breast cancer cell line. Dou et al. [92] found that oligochitosan effectively contribute to the apoptosis of HL-60 and similar behavior has been observed by Xu et al. [93] for chitooligosaccharides when used for human hepatocellular carcinoma cells (SMMC-7721 cells). Kumar et al. [94] studied the anticancer and antimicrobial effect of a new chitosan thymine conjugate prepared via acylation reaction between chitosan and thymine-1-yl-acetic acid and reported that the chitosan thymine conjugate considerably decreased the production of tumorous human liver hepatic cellular carcinoma cell line HepG2 cells with non-cytotoxic effect. Positive results of using chitosan as anticancer material have been reported by Hossain and Takahashi [95] and Hwang et al. [96] for improving the apoptosis in the human colon adenocarcinoma, HT-29 cells and prevent tumor of mouse monocyte-macrophage, RAW 264.7 cell line respectively. It has also been used for bladder cancer [97] and colon cancer [98]. Yamada and Clark [99] investigated the effect of chitosan on the Ehrlich ascites tumor (EAT) cells via nucleosomal DNA obliteration. Chitosan has also been used for prevention as proposed by Shen et al. [100]. They showed that chitosan has negative influence on the increase of HepG2 cells and destroys cancer cell growth in HepG2-carrier mice.

The amino group in chitosan under acidic medium is responsible for the mucoadhesive properties and increase the residual time at the absorption site. This makes chitosan nanoparticles an effective carrier for the oral absorption of drugs [101, 102]. Ringdorf [103] introduced the concept of using polymer nanoparticles for delivering small molecule drugs to their site of action in 1975. Based on this concept, several works successfully applied to use chitosan-anticancer drug conjugates, such

as doxorubicin conjugated glycol chitosan (DOX-GC) with a cis-aconite spacer [104] and n-lauryl–carboxymethyl which was used as carrier for hydrophobic cancer drugs [105].

The chitosan-based nanoparticles were found to accumulate preferentially in the tumor tissue known as EPR (enhanced permeability and retention) effect [105]. These chitosan byproducts have the capacity to solubilize taxol micelles to be therapeutically useful. Another chitosan-based polymeric micelle for taxol delivery in cancer therapy has been tested by Zhang et al. [106]. They obtained new N-alkyl-O-sulphated chitosan that could be considered as useful taxol for drug carrier. N-succinyl-chitosan derivatives conjugated have also shown good antitumor activities with mitomycin C (MMC) [107]. These chitosan-based conjugates were also proven to have good action against murine leukemia (L1210 and P338), B16 melanoma, murine liver metastatic tumor (M5076, Sarcoma 180 solid tumor) and murine hepatic cell carcinoma (MH134). Tan et al. [108] reported interesting results of chitosan as anticancer drug conjugates. This is attributed to the excellent properties of chitosan, and it use as nanoparticle for drug delivery systems.

1.3.2 Antimicrobial Activity of Chitosan

Many antimicrobials materials such as sulfite, benzoate, sorbate were employed as reliable stabilizers to control microbial contaminants and risks in food manufacturing for decades [109]. These synthetic materials are not considered healthy foods; consumers are looking for safer and preferably natural antimicrobials. Chitosan has attracted much attention as the natural substitution material and offers a wide variety of antimicrobial activity including bacteria, yeast, and fungi [110]. Moreover, it exhibits more advantages relative to other natural antimicrobials, such

as better antimicrobial properties, a wider range of the behavior spectrum, greater killing extent and minor mammalian cells toxicity [110].

Several mechanisms can explain the antimicrobial activity of chitosan. Nikaido [111] reported that the gram-negative bacteria are more resistant to amphiphilic and lipophilic inhibitors compared with the gram-positive bacteria. Chitosan cannot pass through the external layer of gram-negative bacteria. However, the positive charge of the chitosan molecule which comes from the amino group at C-2 below its pK_a (pH 6.3), creates the polycationic structure of chitosan. This polycationic structure interacts with the predominantly anionic components of the gram-negative surface such as proteins and lipopolysaccharides. According to Helander et al. [112] the polycationic chitosan binding of outer membrane (OM) of gram-negative bacteria caused to interrupt and loss barrier function of the OM. The antibacterial activity of chitosan obtained from the interface of chitosan's positive charges with the macromolecules negative charges at the bacterial cell surface, which changes the cell permeability [113, 114].

Sudarshan et al. [114] studied the mechanism of antibacterial activity of chitosan by referring to the binding of the amino group of chitosan to the surface components of bacteria. Chitosan cause cell death due to leakage of intracellular components at lower concentrations (0.1 mg mL^{-1}). Additional coating of the bacterial surface can be achieved at a higher level (2.0 and 5.0 mg mL^{-1}). This effect can stop the leakage of intracellular components as well as to impede mass transfer across the cell barrier [115]. Helander et al. [112] reported experimental confirmation about the mechanism of antibacterial activity of chitosan namely *E. coli*, *P. aeruginous* and *S. Typhimurium* by using electron microscopy. It is clear that chitosan disrupts the barrier properties of the outer membrane of gram-negative

bacteria. The electron micrographs demonstrated that site action of the chitosan is at the outer membrane (OM) of bacteria. Similar experimental results were stated by Coma et al. [115] for the bactericidal activity of chitosan on bacteria like *L. innocua*. They attributed the action to the interface of positively charged of chitosan with the negatively charged of the bacterial cell surface.

Previous studies on the activity of chitosan with biological membranes cannot sufficiently explain the electrostatic interactions of the protonated amino groups at low pH [116]. Another study mentioned that hydrophobic interactions have an important role in the polysaccharide action. In the investigation, the effect of pH and molecular weight of chitosan showed that increasing molecular weight (213 kDa) and decreasing pH can lead to disruption of the membrane [117]. The ionization of the amino groups of chitosan increases the antimicrobial activity of chitosan with decreasing pH to below 6 [10, 19, 23]. At pH 7, unmodified chitosan is not antimicrobial active because it does not dissolve and does not contain any positive charge [21, 118]. The antimicrobial activity of water-soluble chitosan derivatives depended on the degree of deacetylation and the substituted group [118].

A study has shown that anti-fungal and bactericidal activities of chitosan can be due to the ability of chitosan to interrupt the inner and outer membranes of the cells [119]. The role of electrostatic, hydrophobic and hydrogen bond in the interactions between chitosan and three different lipids has been investigated using Langmuir films to mimic the interaction between chitosan and bacterial membranes [120]. The results showed that the effect of chitosan on dipalmitoyl phosphatidylglycerol (DPPG) can be negligible [120]. This effect was found to decrease with increasing pH, due to the charge-mediating action of chitosan, whereas

pH did not affect the cholesterol monolayers where interactions occur mainly via hydrogen bonding.

1.3.2.1 Determination of Chitosan Antimicrobial Activities

Antimicrobial activities of chitosan were obtained by three methods, namely agar dilution, broth microdilution and disk diffusion. These techniques are recommended by Clinical and Laboratories Standards Institute (CLSI) as standard methods *in vitro*, to determine the sensitivity of bacteria applied as antimicrobial agents (CLSI, 2009; CLSI, 2009) [121]. Disk diffusion technique tests a large number of antimicrobials separately in a comparatively easy and cheap way and the results can be considered as qualitative since it can only show the sensitivity of antimicrobials against the bacteria tested, which is defined as susceptible, intermediate, and resistant that is associated with diameter of inhibition region. The disk diffusion technique can not determine the minimum inhibitory concentration (MIC), also it is hard to test the susceptibility of fastidious and slow-growing bacteria [122]. Besides, it is labor-intensive and time-consuming, in the same manner as other agar-based techniques [123].

Many reported works used disk diffusion to define the antimicrobial activities of chitosan [123-126]. Moreover, this technique is not always dependable for determining the antibacterial activity of natural antibacterial due to the polarity of the natural compounds that can affect the diffusion of compounds onto the culture medium [15].

Agar dilution technique is a quantitative susceptibility testing method using two-fold dilutions of an antibiotic made in Mueller-Hinton agar (MHA) medium and after that bacterial suspensions were inoculated on MHA [121].

The advantages of this method are that it can investigate the weaknesses of a number of bacteria in one plate and can test the fastidious microorganisms, and the agar can upkeep the bacteria growth sufficiently at the same time. Moreover, the agar dilution method is not usually used in most microbiology laboratories as it is costly, need intensive work and time-consuming. Broth microdilution technique is also a quantitative method normally employed in medical test centers and laboratories. This technique can also investigate the effects of numerous antimicrobials at the same time. Additionally, this method is highly saving in media usage, requires a small amount of testing sample and less time-consuming compared to the agar-based technique [127].

Both agar dilution and broth microdilution techniques can be applied to define MIC value. Earlier work reported that broth microdilution showed less MIC values than agar dilution for 47 gram-negative bacteria [127]. However, many studies have been done to compare the agar dilution and broth microdilution in antibiotic susceptibility testing and found a real association between them [127, 128]. Luber et al. [127] suggested that the broth microdilution method would be suitable and fast screening method for MIC determination as compared to the other methods. Based on a rather recent study, broth microdilution method can be recommended as a quick screening method for MIC determination. However, presently there are few studies on the evaluation of methodologies used for measuring the antimicrobial activity of chitosan, indicating that continuation of our study is needed for future work. However, in this research, MIC was determined by the broth microdilution method.

A broad range of MIC for chitosan to be used as antimicrobial material ranging from 0.005 % to 1.5 % (w/v) [129, 130, 131] has been reported. High

concentration of chitosan (1-1.5 %) showed complete inhibition of *S. aureus* after two days of incubation [132], while in another work chitosan concentrations lower than 0.005 % was enough to achieve deactivation of *S. aureus* [131]. To hamper the growth of *B. cereus* 0.02 % of chitosan was considered necessary as stated by Simpson et al. [132], Daramdji and Izumimoto [133] reported that for *E. coli* inhibition 0.1 % concentration of chitosan in meat protection, while another work reported that 0.0075 % of chitosan was adequate to stop the intensification of *E. coli* [131]. Moreover, at pH 5.5 chitosan with concentrations of 0.5 or 1 % can totally deactivate the growth of *E. coli* [134]. Besides, No et al. [130] reported that the growth of *L. monocytogenes* and *V. para haemolytic* us were completely inhibited by applying 0.1 % concentration of chitosan with molecular weight of 746 kDa and 470 kDa, respectively.

1.3.2.2 Factors Affecting the Antimicrobial Activity

Several factors affect the antimicrobial activity of different chitosans. These factors include two categories, i.e., intrinsic and extrinsic factors, such as Mw, DD, concentration, viscosity, solvent, pH, test strains, temperature and metal ions [3]. Moreover, the variation of application methodologies is an additional important factor that changed the results of chitosan antimicrobial activity.

1.3.2.2.1 Intrinsic Factors

In spite of numerous investigations the intrinsic and extrinsic factors influencing the activity of chitosan in antimicrobial applications, it is still a great challenge to identify the effect of the DD or molecular weight on this activity. For example, chitosan showed greater antimicrobial competence against gram-negative